4.

Quantitative Serologic Relationships Within the Artiodactyla.[1]

Joseph G. Baier, Jr. and Harold R. Wolfe

Department of Zoology, University of Wisconsin

(Text-figure 1).

Introduction.

Any study of animal relationships justifies the use of a serological method capable of stating in exact mathematical terms the degree of relationship of the animals being studied. In the present study a volumetric quantitative precipitin technique having these qualifications was employed in a study of the blood sera of some Bovidae and Cervidae.

Although the precipitin reaction has been used in the study of plant and animal relationships since 1900, only a few investigators have thought of its use in exact quantitative terms. Boyden (1926) expressed its quantitative nature and stated that measurements of degree of relationship so obtained are independent of interpretation. Further elaborating on the idea, Boyden & Baier (1929) devised an exact quantitative volumetric precipitin technique that is "simpler and more rapid than any other which has been used in the quantitative study of blood relationships, and that through it highly significant *measurements* of biological relationships may be made." Their technique of measuring volumetrically the amount of precipitate formed in the reaction was a decided improvement over the methods of Nuttall (1904), Schur (1904), Hamburger (1905), and Mollison (1924), in that of all these workers, they were the only ones who gave an adequate statement of the reliability of their technique. In one series of 36 determinations the average error of the individual readings was 5 per cent. when compared with the mean of the series. The average deviation of the means of successive pairs of readings was the same as the deviation of the whole series, while the means of the values taken in quartets dropped to 3 per cent. An error in technique of this value, when supported by statistical analysis, is indeed very significant. According to Boyden (1934), "the results of the application of such a technique to the study of serologic relationships should be of great interest. It is likely that this technique will succeed in distinguishing closely related species, which have heretofore been indistinguishable by the precipitin test." So far, this is the only volumetric test which has been used in serological relationships.

Using this improved volumetric technique, Baier (1933) established the constancy of in-vitro factors for proper execution of the tests. Wolfe & Baier (1938) by using the ring test and the volumetric precipitate measurement procedure showed that the in vivo injection procedure may influence the "type" of precipitin that may be produced. They found that high-titered (ring test) antisera were produced by one or two series of injections of undiluted antigen while continued re-injections resulted in an increase in the precipitate forming power of an antiserum without causing an increase in the ring test "titer" of the antiserum. They indicated the presence of (1) a "titer"-producing antibody and (2) a "precipitate"-forming antibody. It is important when attempting volumetric relationship studies that an antiserum be employed having high precipitate forming powers.

A more recent technique of precipitate measurement which should parallel the volumetric technique is that of the Libby Photronreflectometer (Libby, 1938) which measures the amount of precipitate formed in the precipitin reaction by nephelometric methods. So far two papers have appeared (Boyden, 1938, and DeFalco, 1941) indicating its possible use in relationship studies.

With the reliability of the volumetric technique well established, an investigation

[1] The Works Progress Administration furnished personnel assistance under the University Natural Science Project, Work Project No. 10324 for this research.

The authors wish to thank the various contributors listed in Table I for furnishing the samples of blood sera used in this study.

of the serological relationships of some Bovidae and Cervidae was attempted to parallel a similar study of Wolfe (1939) who reported on some of these same samples of blood. In his paper, however, only the "ring" test was employed.

The earliest work of actual precipitate measurement in a problem of animal relationship studies is that of Nuttall (1904), who with Strangeways reported, among others, some studies made with the bloods of some Cervidae and Bovidae. Since then only the paper of Boyden (1934) reported one brief study of an ox-sheep reaction as a suggestion of the possible use of a volumetric precipitin technique.

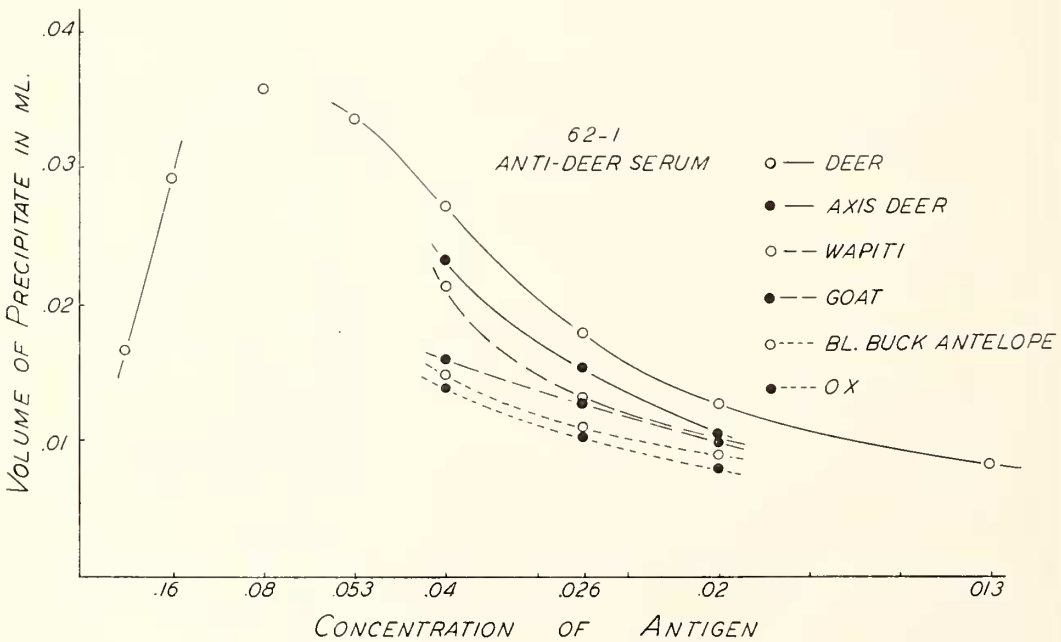
Materials and Methods

Antibodies were produced in healthy adult male and female rabbits of various breeds. The undiluted serum antigens were injected intravenously at intervals of a month or more; the initial series of injections consisted of three injections given on alternate days and the total quantity of antigen injected was 3 ml. Subsequent series of injections consisted of two injections on alternate days of a total of 1.5 ml. This method of antigen injection was shown by the authors (1938) to be conducive to the production of good precipitating antisera.

The animals were bled from the heart with sterile syringes and needles, the blood allowed to clot, and the exuded serum filtered through Seitz filters. The serum was transferred to sterile ampoules and stored in the refrigerator until used.

The serum antigens used for antibody production (Table 1) were deer, ox, buffalo, sheep, and goat. Test antigens consisted of various species of Artiodactyla (Table II).

The tests, in vitro, were carried out following the method of Boyden & Baier (1929) using standardized Van Allen thrombocytocrits to measure volumetrically the precipitate obtained by incubating known amounts of antigen and antiserum. Mixtures of 0.5 ml. of antiserum and 0.5 ml. of antigen (either homologous or heterologous), diluted to give a protein content so as to stay in the range of relative antibody excess, were made directly into the thrombocytocrits. These were then placed in a water bath at 37.5° ± 0.5° C for one hour and centrifuged for two fifteen-minute periods in a tachometer controlled centrifuge at the rate of 2,400 R.P.M. Readings were made in duplicate and sometimes in quadruplicate. The protein content of all antigens was based on total nitrogen which was determined by a modified macro-Kjeldahl method. The test antigens were standardized to give similar protein content by diluting with buffered saline (Evans, 1922). It was necessary at times to dilute the antiserum with buffered saline in order to avoid an excessive amount of precipitate. Tests with any one antiserum were always made at a constant dilution of antiserum. Any variations in the measurable precipitate were due then to differences in the proteins of the various blood sera used in these relationship studies. Relationships were recorded in terms of the percentage of volume given by a heterolog-



ous determination in comparison to the homologous precipitate volume taken as 100 per cent. The average of at least six readings on the curve of reaction, of three different antigen concentrations in the area of relative antibody excess was taken for any one relationship value. By using six readings as a minimum the statistical reliability of the test could be established (Boyden & Baier, 1929) and by taking these readings from the area of relative antibody excess the resolution of the precipitate by excess antigen could be avoided as well as obtaining a greater constancy in the readings (Baier, 1933). He also demonstrated that it is not advisable to use the entire curve of reaction since readings taken in the area of antigen-antibody equilibrium are unreliable. Text-fig. 1, taken at random from Table V, illustrates these points from the reaction of anti-deer serum with the homologous and heterologous antigens used in this study. The region of relative antigen-antibody equilibrium is shown as the discontinuous peak of the curve. To the left is the area of antigen excess, while to the right is shown the area of antibody excess where the heterologous readings were made for relationship studies.

Results.

In Table III are presented the data obtained from two anti-ox and one anti-buffalo sera. These antisera were reacted with their homologous antigens and with several heterologous antigens.

The data of 21–3 and 22–2 show that the buffalo and eland sera are more closely related to ox than are the sera of the other Bovidae or the Cervidae. Furthermore, the per cent. values indicate that buffalo is more closely related to ox than is the eland. This essentially verifies the results of Wolfe (1939), but it should be emphasized that the technique employed in the present paper enables a distinction between ox and buffalo antigens which he could not show using the ring test with unabsorbed sera.

The two ox antisera did not give similar degrees of reaction in per cent. with the heterologous antigens but the relative positions of the animals was constant. Similar results will be noted throughout this paper. It is advisable, therefore, to emphasize

Table I.

Test antigens.

Family	Scientific Name	Common Name	Source of Material
Bovidae	*Bos taurus*	Ox (2 samples)	Mayer Packing Company
	Bison bison	American buffalo	Yellowstone National Park
	Taurotragus oryx	Eland	New York Zoo
	Poëphagus grunniens	Yak	San Diego Zoo
	Anoa depressicornis	Anoa	New York Zoo
	Ovis aries	Sheep (3 samples)	Mayer Packing Company
	Capra hircus	Goat	University Farm
	Ovis tragelaphus	Aoudad	New York Zoo
	Ovis canadensis by Ovis musimon	Mountain sheep hybrid	San Diego Zoo
	Antilope cervicapra	Black buck antelope	New York Zoo
Cervidae	*Odocoileus virginianus*	White-tailed or Virginia deer	Madison Zoo
	Cervus axis	Axis deer	New York Zoo
	Cervus canadensis	Wapiti (2 samples)	New York Zoo

Table II.

Rabbit Number	Antigen Injected	Series of Injections	Quantity Injected: First Series (undiluted)	Quantity Injected: Additional Series (undiluted)	Bled (days following last injection)	Homologous Titer (ring-test)
21–3	Ox	4	3 ml.	1.5 ml.	10	1,024,000
22–2	Ox	3	3 ml.	1.5 ml.	10	256,000
54–2	Buffalo	3	3 ml.	1.5 ml.	10	512,000
30–2	Sheep	3	3 ml.	1.5 ml.	10	512,000
50–3	Goat	4	*	1.5 ml.	10	512,000
51–3	Goat	4	*	1.5 ml.	10	256,000
62–1	Virginia deer	2	*	1.5 ml.	7	512,000
62–2	Virginia deer	3		1.5 ml.	7	512,000
62–3	Virginia deer	4		1.5 ml.	8	512,000
62–4	Virginia deer	5		1.5 ml.	8	512,000
62–5	Virginia deer	6		1.5 ml.	8	512,000

* Between .75 to 1.5 mg. total protein per kg. of body weight. This is actually the second series of injections as the animals were previously injected with minute quantities and reported by Wolfe (1939).

TABLE III.

Antigen	21–3 anti-beef	Antisera anti-beef 22–2	anti-buffalo 54–2
Ox – 1	100.0	100.0	78.8
Buffalo	94.3	84.9	100.0
Eland	84.8	64.17	—
Yak	—	—	77.7
Anoa	—	—	74.4
Sheep – 5	44.9	52.90	—
Sheep – 6	—	54.53	—
Sheep – 6W	—	—	38.4
Goat	36.0	53.03	—
*Black buck antelope	42.2	32.47	22.7
*Wapiti	43.71	42.8	30.7
*Axis deer	50.13	39.23	42.3
Virginia deer	45.8	53.93	44.8

*Data may not be reliable due to excessive hemoglobin in test samples.

phylogenetic position rather than actual per cent. relationship.

The percentages in the reactions of the Bovidae sera, other than the buffalo and eland, and of the Cervidae sera, were, on the whole, quite similar. Thus the wapiti, Virginia deer and axis deer seem to be as closely related to the ox as are the sheep, goat and black buck antelope. Since such closely related forms as the goat and sheep or the axis deer and wapiti did not, as would be expected, give similar percentage reactions, it seems necessary to treat the more distantly related forms as a group, rather than to attempt to give each animal a definite position in the table.

The buffalo antiserum (54–2) was reacted with yak and anoa bloods as well as with some of the antigens tested with ox antisera. The ox, yak, and anoa bloods all gave similar percentages and showed a much closer relationship to buffalo than did the other Bovidae and Cervidae. This result was to be expected. Again the more distantly related forms gave inconsistent results and must be treated as a group.

Table IV presents the data obtained from anti-sheep and anti-goat sera reacted against their homologous antigens as well as representative heterologous antigens. The anti-sheep serum (30–2) was able to distinguish between sheep and goat sera, and their high percentage values indicate a closeness in the relationships of these forms. That these percentage values are statistically reliable is indicated by the ratios of the means to their respective probable errors. For sheep–6 serum the ratio was 55:1, while for goat serum the ratio of the mean to the probable error of the mean was 140:1. These figures, in being well above the 4:1 ratio generally accepted as indicating statistical reliability, are highly reliable in stating that by means of this volumetric test it was possible to distinguish sheep serum from goat serum which had not been hitherto usually possible using the ring test with unabsorbed sera. The order of relationship for the other animals indicated that ox and buffalo were more closely related to the sheep than were the Virginia deer, black buck antelope, eland, axis deer and wapiti. The reactions of the sera of these distantly related forms did not give the consistent re-

TABLE IV.

Antigens	30–2 anti-sheep	Antisera 50–3 anti-goat	51–3 anti-goat
Sheep – 6	100.0	—	—
Sheep – 5	99.60	91.9	63.8
Goat	96.30	100.0	100.0
Aoudad	—	84.3	—
Mountain sheep hybrid	—	81.2	—
Ox – 1	87.40	42.8	27.6
Buffalo	85.57	44.7	24.8
Eland	70.40	—	—
*Black buck antelope	71.57	—	—
Virginia deer	77.27	51.3	30.7
*Axis deer	68.53	40.9	20.5
*Wapiti – 1	61.7	—	23.1
*Wapiti – 2	—	54.8	22.8

* Data may not be reliable due to excessive hemoglobin in test antigens.

sults expected of closely related forms and these species should be regarded as a group rather than individually.

The two anti-goat sera (50–3, 51–3) were more specific than the anti-sheep serum. The sheep serum again could be distinguished from the goat serum, and the high percentage reaction of the aoudad and mountain sheep hybrid show their closeness to the goat. On the other hand, the remaining Bovidae and Cervidae reactions were lower and it cannot be said which ones are more closely related to the goat. This result is consistent with the data for anti-ox and anti-buffalo sera presented in Table III.

Antigoat serum 51–3 gave much lower percentages with the heterologous antigens than did 50–3, indicating that the former serum can be considered to be more specific. Such differences in serum specificity are also known to occur with the ring test method.

Table V illustrates the data of five anti-Virginia deer sera produced in a single rabbit. This rabbit was given several series of injections and bled after each series. Animals injected by this method were shown by Wolfe & Baier (1938) to produce high precipitating antisera useful in quantitative volumetric precipitin studies but giving very aspecific reactions if the ring test technique is used.

The reactions of all five antisera gave larger amounts of precipitate with members of the deer family than with the Bovidae. In every case the axis deer and wapiti could be distinguished readily from the Virginia deer, while the Bovidae gave considerably lower percentage values. The relative closeness of the axis deer and wapiti to the Virginia deer is not definite from the data presented. In two out of three instances where both tests were made the axis deer showed the closer relationship, but in the other just the opposite condition was observed. Then again, the two wapiti bloods do not give the same degree of reaction. The only explanation the authors wish to offer is that these samples of sera contained a large amount of hemoglobin and possibly the per cent. of protein, obtained on the basis of total nitrogen, was inaccurate. The authors feel that exact protein content of the active antigens is essential and disagreements of the type illustrated can be avoided only if better methods can be devised for measuring only the reactable antigens.

Discussion.

The data presented in this paper confirm and extend the evidences for facts concerning the blood relationships of some species of Bovidae and Cervidae previously shown by morphological and by other serological techniques.

That morphology has its place in phylogeny is not disputed; that it has its limitations is evident. The serologist can apply his studies to a more exact and quantitative estimation of present relationships with a technique which is independent of morphology. This is the aim of the present paper.

The volumetric method of measuring the amount of precipitate formed in the precipitin test has enabled a distinction between some very closely related forms. Thus ox and buffalo, and sheep and goat could be distinguished from each other. This is usually not possible with unabsorbed antisera using the ring test. The advantages of the volumetric test over the ring test is of this nature. Its disadvantages are that the test is time-consuming in its operation, protein contents of all test antigens must be very accurately determined, and as to the data, it has not been possible so far to indicate the degree of relationship of more distantly related forms. It is hard to explain why the more distantly related forms reported in this paper show inconsistent degrees of relationship to a test antiserum and why all of these distantly related forms, regardless of their phylogenetic position, show about the same per cent. of distant relationship. No attempt was made to study

Table V.

Antigen	Anti-Virginia Deer Sera				
	62–1	62–2	62–3	62–4	62–5
Virginia deer	100.0	100.0	100.0	100.0	100.0
*Axis deer	83.7	—	96.4	72.4	59.6
*Wapiti – 1	76.2	66.3	88.6	—	66.4
*Wapiti – 2	—	77.0	—	—	78.1
Goat	69.1	43.7	76.8	56.8	—
Sheep – 5	—	45.9	77.2	69.6	50.6
Ox – 1	—	44.7	70.9	—	48.8
Ox – 5	57.1	—	—	—	—
Buffalo	—	41.6	76.8	63.5	53.2
Eland	—	40.6	—	50.9	—
*Black buck antelope	60.9	37.3	61.9	59.0	—

* Data may not be reliable due to excessive hemoglobin in the test antigens.

this problem at the present time; distantly related forms were merely spoken of as a group rather than as individuals.

A possible explanation may be that the relative position of the region of antibody-antigen equilibrium in shifting toward the left (refer to Text-fig. 1), as reactable antigen decreases and toward the right as reactable antigen increases will alter the nature of the curves being studied, and only by studying antigens having approximately the same reactable antigen content will the curves of reaction be reliable. No degree of controlling total antigen content by means of Kjeldahl determinations can alleviate this difficulty as the Kjeldahl determinations measure the total protein content of test antigens and not the per cent. of reactable or specific protein antigens.

The ring test titers on the other hand, are not influenced either by slight variations in protein concentration or by the proportions of antigen and antibody in the reaction mixtures since the end point used (titer) is simply the maximum dilution of an antigen that will form a ring of precipitate at the junction of antigen and antibody.

Comparisons of the data obtained by the ring test and by the present volumetric technique can be made by referring to the paper of Wolfe (1939), who reported on three of the rabbits used to produce antisera in the present paper. These rabbits (numbers 50, 51, and 62) were given one series of minute injections of antigen to produce the specific antisera required to distinguish closely related forms using the ring test, and were then given additional series of larger injections to produce the high precipitate forming antisera for use in the volumetric precipitin test. The results are in general agreement as has been stated previously.

The technique employed has consistently enabled a distinction of such closely related forms as ox from buffalo, sheep from goat, and Virginia deer from axis deer and wapiti. Were the ring test to be employed, such distinctions could not be consistently made with undiluted sera. The volumetric method used in this paper seems to be a very reliable one in showing the differences of very closely related forms. On the other hand, the more distantly related forms used in this work can be classified together only as a group rather than as individuals. A more or less rough grouping is possible from the data presented. The more closely related forms could be placed in one group and subdivided according to their closeness of relationship while the more distantly related forms were placed in a second group and subdivision was not possible except in one instance (anti-sheep serum).

In tabular form, this data can be presented as a brief serological classification in the following fashion.

A. Classification based on anti-beef sera
   - Group I
     - A. Ox
     - B. Buffalo
     - C. Eland
   - Group II Other Bovidae and Cervidae tested

B. Classification based on anti-buffalo serum
   - Group I
     - A. Buffalo
     - B. Ox, yak, anoa
   - Group II Other Bovidae and Cervidae

C. Classification based on anti-sheep serum
   - Group I
     - A. Sheep
     - B. Goat
   - Group II
     - A. Ox, buffalo
     - B. Other Bovidae and Cervidae

D. Classification based on anti-goat sera
   - Group I
     - A. Goat
     - B. Sheep
     - C. Aoudad, mountain sheep hybrid
   - Group II Other Bovidae and Cervidae

E. Classification based on anti-deer sera
   - Group I
     - A. Virginia deer
     - B. Axis deer and wapiti
   - Group II Bovidae

Summary.

1. The sera of thirteen representative species of Bovidae and Cervidae were used as test antigens.
2. Eleven antisera were produced against five of these thirteen species.
3. Antisera were produced having high precipitate forming powers.
4. The per cent. of relationship is reported on the basis of the volume of precipitate formed in the reaction mixture when compared with the homologous reaction taken as 100%.
5. Ox, buffalo and eland are related to each other in the order named, and could be distinguished from each other.
6. Virginia deer could be distinguished from axis deer and wapiti.
7. Ox, yak and anoa are closely related to buffalo.
8. Sheep and goat could be distinguished from each other.
9. Aoudad and mountain sheep hybrid could be distinguished from goat and these forms were more closely related than were the other Bovidae.
10. This work confirms ring test studies, but enables a finer distinction of closely related forms.

Bibliography

Baier, Joseph G., Jr.
1933. Quantitative studies on precipitins. *Physiol. Zool.*, 6:91.

Boyden, Alan A.
1926. The precipitin reaction in the study of animal relationships. *Biol. Bull.*, 50:73.

Boyden, A. A.
1934. Precipitins and phylogeny in animals. *Am. Nat.*, 68:516.

Boyden, A. A.
1938–39. Serological study of the relationships of some common invertebrata. Carnegie Inst. Wash. Year Book No. 38:219.

Boyden, A. A., & Baier, J. G., Jr.
1929. A rapid quantitative precipitin technique. *Jour. Immun.*, 17:29.

DeFalco, R. J.
1941. A comparison of antigens by interfacial and nephelometric methods. *Proc. Soc. Exp. Biol. and Med.*, 46:500.

Evans, A. C.
1922. A buffered physiological salt solution. *Jour. Inf. Dis.*, 30:95.

Hamburger, H. J.
1905. Zur Untersuchung der quantitativen Verhältnisse bei der Präzipitinreaktion. Folia haemotologia, 2:539.

Libby, R. L.
1938. The photronreflectometer—an instrument for the measurement of turbid systems. *Jour. Immun.*, 34:71.

Mollison, T. H.
1924. Serodiagnostik als Methode der Tiersystematik und Anthropologie. Abderhalden's Handbuch der biologischen Arbeits-Methoden, Abt. 9, Teil 1, Hälfte 1, p. 554.

Nuttall, G. H. F.
1904. Blood immunity and blood relationship. Cambridge, England: Cambridge University Press.

Schur, H.
1904. In: Kolle and Wasserman. Handbuch der pathogenen Micro-organismen, 4:631.

Wolfe, H. R.
1939. Serologic relationships among Bovidae and Cervidae. *Zoologica*, 24:309.

Wolfe, H. R., & Baier, J. G., Jr.
1938. Comparison of quantitative precipitin techniques as influenced by injection procedures. *Physiol. Zool.*, 11:63.